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The 5′ and 3′ extremities of the satellite tobacco necrosis virus translational enhancer domain contribute differentially to stimulation of translation

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ABSTRACT

The translational enhancer domain (TED) of satellite tobacco necrosis virus (STNV) RNA stimulates translation of uncapped RNAs autonomously. Here we set out to identify the 5′ and 3′ extremities of TED and features of these sequences with respect to translation. We found that both in wheat germ extract and in tobacco protoplasts, the 5′ border is confined to 3 nt. Mutational analysis revealed that the autonomous function of TED is sensitive to 5′ flanking sequences. At the 3′ end of TED, 23 nt have a cumulative, quantitative effect on translation in wheat germ extract, whereas in tobacco protoplasts, the most 3′ 14 nt of these 23 nt do not enhance translation. The 5′ and 3′ sequence requirements triggered the development of a new secondary structure model. In this model, TED folds into a phylogenetically conserved stem-loop structure in which the essential 5′ nucleotides base-pair with the 3′ nucleotides that stimulate translation both in vitro and in vivo. Importantly, the 14 3′ nucleotides in TED that stimulate translation in the wheat germ extract only do not require the predicted base-pairing in order to function. The discrepancy between in vitro and in vivo sequence requirements thus correlates with potential base-pairing requirements, opening the possibility that TED contains two functional domains.

Keywords: 3′ UTR; cap-independent translation; plant virus; RNA secondary structure; tobacco; wheat germ

INTRODUCTION

Translation initiation in eukaryotic cells requires, as a first step, the recognition of the mRNA by the translational machinery. In most cases, this implies recognition of the 5′ cap structure by initiation factor (eIF)4E (Hershey & Merrick, 2000). A different way of recruiting the translational machinery is used by the translational enhancer domain (TED) of the uncapped satellite tobacco necrosis virus (STNV) RNA (Danthinne et al., 1993; Timmer et al., 1993). TED stimulates translation synergistically with the STNV leader. Moreover, TED is sufficient to stimulate translation of uncapped, heterologous RNAs autonomously. Although it is known that TED locates within a 122 nt sequence in the 3′ UTR of STNV-2 (Danthinne et al., 1993), the sequence and structural requirements for TED function have not been studied in detail.

It is possible that TED uses features similar to other *cis* elements that promote cap-independent translation. RNA sequences of which primary and secondary structure requirements for translation have been studied include the internal ribosome entry sites (IRES elements; Belsham & Jackson, 2000) and the 3′ UTR sequences of the uncapped RNA of barley yellow dwarf virus (BYDV; Wang & Miller, 1995). An apparent homology between functional elements in the IRESs and the 3′ TE is a sequence stretch containing complementarity to the 3′ end of the 18S rRNA (Pilipenko et al., 1992; Wang et al., 1997; Guo et al., 2000). Importantly, a sequence complementary to the 18S rRNA is also present in TED (Danthinne et al., 1993). 18S rRNA complementarity is, however, insufficient for translation

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stimulation (Tranque et al., 1998; Verrier & Jean-Jean, 2000), and both the IRES elements and the BYDV 3′ TE indeed require specific additional primary and secondary structures for translation stimulation (Stewart & Semler, 1997; Wang et al., 1997; Guo et al., 2000). These elements however do not have obvious similarities to TED. It is thus likely that, although 18S rRNA complementarity may be important, TED requires additional primary and secondary structure information to stimulate translation. In this study, we started to unravel the sequence requirements for the autonomous function of TED by identifying the 5′ and 3′ boundaries of TED and their contribution to translation.

RESULTS AND DISCUSSION

The 5′ border of TED is located between nt 655 and 658

The initial mapping of TED (Danthinne et al., 1993) was based on the availability of restriction sites in the STNV-2 cDNA. The 5′ border of TED was located between nt 632 and 688, and the 3′ border was mapped between nt 691 and 753. The STNV-2 domain with TED function has thus a maximal length of 122 nt. This domain comprises the first stem-loop in the secondary structure model of the STNV trailer (Danthinne et al., 1991; Fig. 3A). As a start to identifying which sequences within this 122-nt domain play a role in cap-independent translation, we determined the location of the 5′ and the 3′ border of TED with respect to its autonomous function more precisely. To this end, TED 5′ and 3′ deletion mutants were generated, and chimeric *cat* RNAs were synthesized with the Ω fragment from tobacco mosaic virus (TMV) (Meulewaeter et al., 1998a) or a 19-nt polylinker sequence ("vector-derived leader"; Meulewaeter et al., 1998b) as leader, and with the TED derivatives as trailer.

In the first series of experiments, the 5′ border of TED was determined in the wheat germ extract using chimeric *cat* RNAs with the vector-derived leader and 5′ TED deletion derivatives. Previous studies showed that removal of nt 655–657 from the 5′ side of TED reduced the translational output of the RNA (van Lipzig et al., 2001). As the autonomous function of TED in wheat germ extract is characterized by a stimulation of the translational efficiency, and not of the functional half-life of uncapped RNAs (Meulewaeter et al., 1998b), we now determined these two parameters from the protein accumulation profiles as described by Danthinne et al. (1993). Figure 1A summarizes the results of five rounds of 5′ deletion analyses. Deletion of the 5′-most 23 nt did not reduce the translational efficiency. Further removal of the single nt 655 reduced the translational efficiency twofold, and removal of two more nucleotides reduced the translational efficiency to levels similar to that for RNA without TED. To ensure that the deletions did not destroy the general translational capacity, capped versions of the RNAs with the TED mutants were tested, and were found to translate with an efficiency similar to capped RNAs lacking TED (data not shown). We therefore conclude that in the wheat germ extract, the 5′ border of TED is located between nt 655 and 658, and the presence of each of the individual nt 655, 656, and 657 contributes to TED function.

In 5′ deletion mappings, the deleted sequences are replaced by adjacent upstream sequences. In the case of removal of the essential nt 655–657 of TED, an AGG triplet is actually replaced by the upstream-located AGC triplet. To investigate whether it is the AGG-to-AGC substitution that causes the loss of function of TED, we synthesized a TED derivative in which the AGG of nt 655–657 was replaced by AGC, not by deletion, but by replacement ($TED_{(657C)}$) and tested the functionality of this TED derivative in wheat germ extract. Figure 1B shows that the translational efficiency of the RNA that contained $TED_{(657C)}$ was about twofold lower than that of the RNA with $TED_{(655–753)}$. This shows that a single nucleotide substitution of G to C at position 657 reduces translational efficiency twofold. The translational efficiency of the RNA with $TED_{(657C)}$ was still 5.5-fold higher than that with $TED_{(658–753)}$. These results show that the lack of function of $TED_{(658–753)}$ is only partially explained by the G-to-C substitution of nt 657, and thus that the autonomous function of TED is sensitive to *cis* sequences outside of TED. A candidate *cis* sequence is the translation stop codon, located only 6 nt upstream. In line with the proposed model for TED function, in which TED recruits the 40S ribosomal subunit upon translation termination (Meulewaeter et al., 1998b), it is possible that the process of translation termination interferes with binding of the 40S ribosomal subunit to TED when TED is located too close to the stop codon.

Having determined the 5′ sequence requirements for TED in the wheat germ extract, we subsequently tested whether these requirements coincide with those in tobacco in vivo. Chimeric *cat* RNAs with 5′ TED deletion derivatives were introduced in tobacco protoplasts, and the CAT protein peak level was determined (Meulewaeter et al., 1998a). To allow for a more reliable quantification of the synthesized CAT protein, RNAs that contain the TMV leader rather than the vector-derived leader were used, as this leader stimulates translation of uncapped RNAs to some extent (Gallie et al., 1987). Moreover, the effects of TED derivatives on translation in the wheat germ extract are similar for RNAs with the TMV leader and the vector-derived leader (data not shown).

Figure 1C shows that deletion of 23 nt at the 5′ end of TED reduced CAT synthesis about twofold in tobacco protoplasts. A further deletion of nt 655–657 reduced CAT synthesis to levels similar to those observed for RNAs with $TED_{(688–753)}$ or a control trailer. The levels of the *cat* RNAs in the protoplasts were similar for

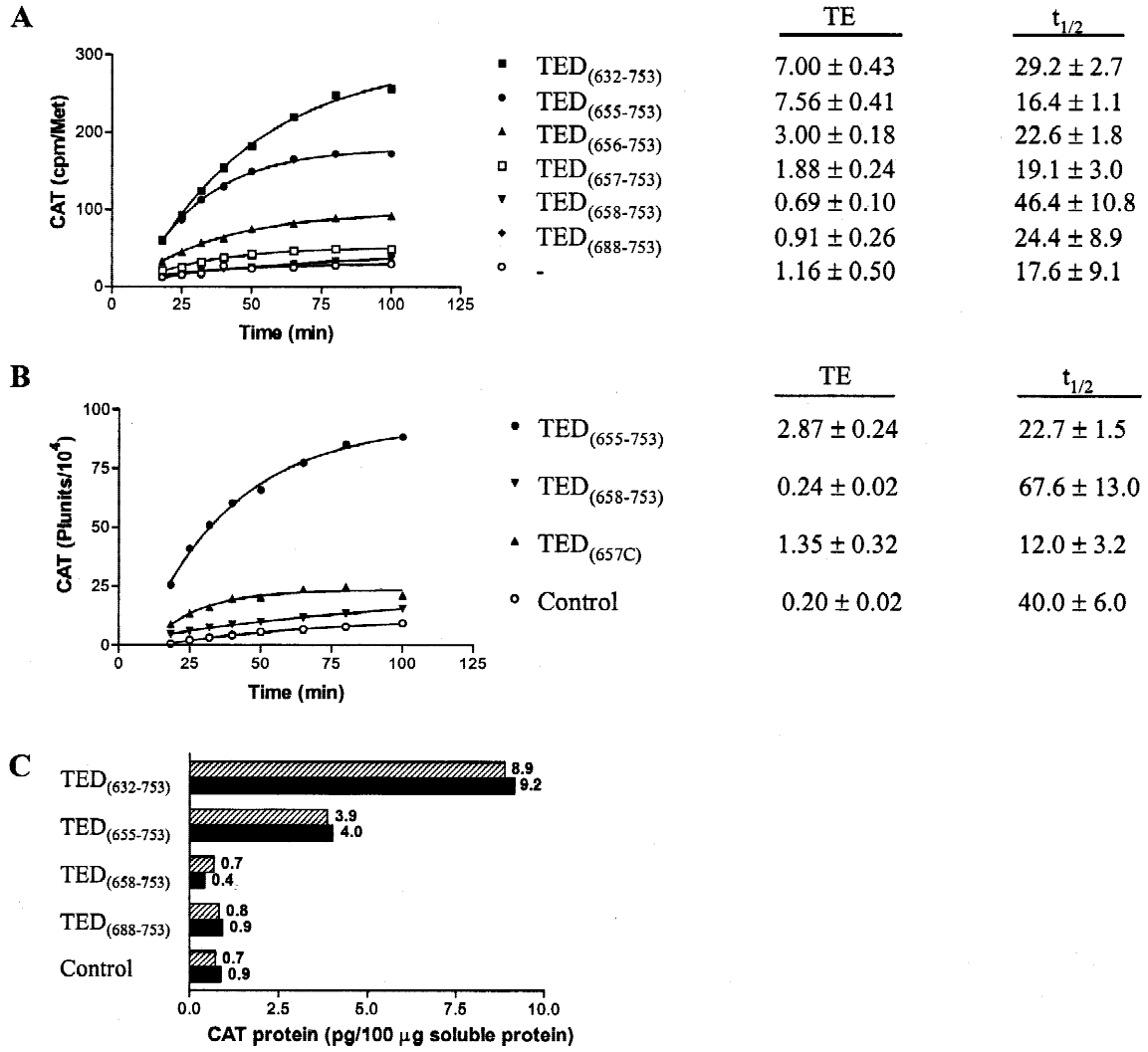


FIGURE 1. The 5′ border of TED is located between nt 655 and 658. **A**: The individual nt 655, 656, and 657 contribute to TED function in wheat germ extract. Translation of uncapped *cat* RNAs with a vector-derived leader and with different 5′ TED deletion derivatives or without trailer (−) in wheat germ extract. The numbers within parentheses indicate the STNV-2 nucleotides, according to their position in the full-length STNV-2 RNA, that are present in the TED derivatives. The translational efficiency (TE) is given in cpm/Met·min, and the functional half-life ($t_{1/2}$) in minutes. **B**: TED 5′ sequences promote translation through both nucleotide composition and positioning of TED. Uncapped *cat* RNAs with a vector-derived leader and, as trailer, different TED derivatives were translated in wheat germ extract. Control indicates that a 120-nt plasmid-derived sequence (Meulewaeter et al., 1998a) is used as trailer. $TED_{(657C)}$ is identical to $TED_{(655–753)}$ apart from a substitution of the G at position 657 by a C. The translational efficiency (TE) is given in PhosphorImager units/10^4·min, and the functional half-life ($t_{1/2}$) in minutes. **C**. Nucleotides 655–657 are essential for TED function in tobacco protoplasts. Uncapped *cat* RNAs with the Ω fragment of TMV as used by Meulewaeter et al. (1998a) and different TED derivatives or the control trailer were introduced in tobacco protoplasts. The CAT protein levels were determined 335 min (hatched bars) and 445 min (black bars) after RNA introduction.

the different TED derivatives (data not shown). The differences in CAT protein synthesis are thus not a consequence of differences in RNA introduction into the protoplasts or in RNA stability. This implies that the CAT protein levels as shown in Figure 1C reflect the translational capacities of the different RNAs. Sequences between nt 632 and 655 thus have the capacity to enhance translation, an effect that was also observed in some experiments in the wheat germ extract (data not shown). Furthermore, nt 655–657 appear essential for TED function in tobacco protoplasts.

Taken together, these results show that the 5′ border of TED in wheat germ extract and in tobacco protoplasts is strict, as it is determined by nt 655–657. This finding is consistent with the observation that nt 655–657 affect the affinity for two wheat germ proteins, binding of which correlates with translation stimulation (van Lipzig et al., 2001).

Sequences downstream of the predicted stem-loop structure are required for maximal TED function

In the next series of experiments, the 3′ TED sequence requirements were determined in more detail. First, the capacity of a series of 3′ TED deletion mutants to stimulate the translational efficiency in the wheat germ extract was tested. Figure 2A shows that progressing 3′ TED deletions in the region nt 738–753 caused a gradual reduction of the translational efficiency. As removal of these nucleotides did not destroy the translational capacity of capped RNAs (data not shown), these data show that nt 738–753 have a cumulative, quantitative effect on TED function.

The quantitative effect of the 3′ sequences on translation opens the possibility that 3′ extensions of TED would stimulate translation further. We therefore tested the functionality of 3′ extended TED versions in wheat germ extract. Figure 2A shows that the RNA with $TED_{(632–760)}$ was translated 20% more efficiently than that with $TED_{(632–753)}$, showing that nt 754–760 further enhance translation. The translational efficiency of the RNA with $TED_{(632–764)}$ and with $TED_{(632–768)}$ was again reduced as compared to that with $TED_{(632–760)}$. In summary, these data show that progressive inclusion of nt 738–760 has a cumulative, quantitative effect on translation in wheat germ.

A quantitative effect of 3′ sequences can be explained in different ways. First, the 3′ sequences could be directly involved in binding to host factors. More binding sites would then increase the number of proteins bound, resulting in a progressive increase in translation, as is suggested for binding of poly(A) binding protein to the poly(A) tail (Munroe & Jacobson, 1990). Alternatively, TED 3′ sequences may progressively increase the affinity for a certain *trans*-acting factor, for example, by inducing conformational changes of the RNA structure.

We next investigated whether the 3′ sequence requirements for TED function in tobacco protoplasts are similar to the requirements in the wheat germ extract. We therefore determined the functionality of $TED_{(632–737)}$, $TED_{(632–746)}$, $TED_{(632–753)}$, and $TED_{(632–760)}$ in tobacco protoplasts. Figure 2B shows that $TED_{(632–746)}$, $TED_{(632–753)}$, and $TED_{(632–760)}$ directed synthesis of similar amounts of the CAT protein. The CAT synthesis from RNAs with $TED_{(632–737)}$ was about threefold lower. RNA quantification showed similar *cat* RNA levels in the protoplasts for the different RNAs (data not shown). The differences in CAT protein levels are thus not due to differences in *cat* RNA introduction or stability. These results therefore show that the 3′ border for maximal TED activity in tobacco is located between nt 737 and 746, and that the contribution of nt 747–760 to translation is minimal. The 3′ essential sequences in the wheat germ extract and in tobacco protoplasts are thus very similar. However, nt 747–760 reproducibly stimulate translation in wheat germ extract and not in tobacco protoplasts.

Taken together, these observations reveal two differences between the 5′ and the 3′ border of TED. First, the 5′ border is determined by only 3 nt, whereas at the 3′ end, 23 nt have a cumulative, quantitative effect on translation in wheat germ extract. Second, the 5′ nt 655–657 are essential in both wheat germ extract and tobacco protoplasts, whereas the 3′ nt 747–760 only stimulate translation in wheat germ extract. This latter difference suggests that *trans* requirements for the 5′ TED sequences appear to have a similar availability in wheat germ extract and in tobacco protoplasts, whereas

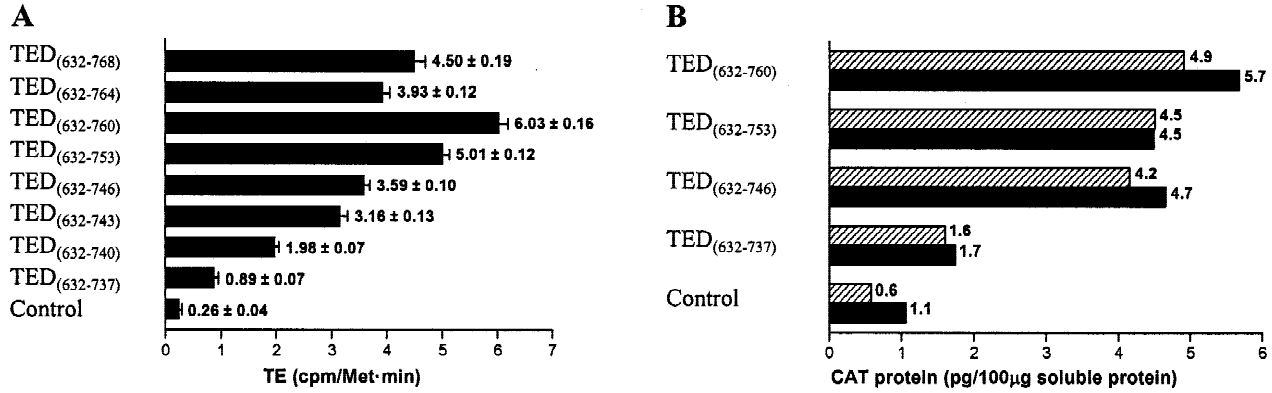


FIGURE 2. Sequences downstream of nt 737 are required for TED function. **A**: Nucleotides 738–760 have a cumulative, quantitative effect on translation in the wheat germ extract. Uncapped *cat* RNAs with a vector-derived leader and progressive 3′ TED deletion derivatives as trailer or the control trailer were translated in the wheat germ extract. The translational efficiency (TE) is given in cpm/Met·min. **B**: The 3′ border of TED for maximal activity in tobacco protoplasts is located between nt 737 and 746. Uncapped *cat* RNAs with the TMV leader and different TED derivatives or the control trailer were introduced in tobacco protoplasts. The CAT protein levels were determined 275 min (hatched bars) and 400 min (black bars) after RNA introduction.

trans requirements of nt 747–760 have a different availability in these two systems. This may imply that the *trans* requirements of the 5′ sequences are different from those of the 3′ sequences 747–760.

Another feature of the 3′ required sequences, nt 738–746, is that they are located downstream of the stem-loop structure predicted by Danthinne et al. (1991) (Fig. 3A). This shows that this predicted stem-loop struc-

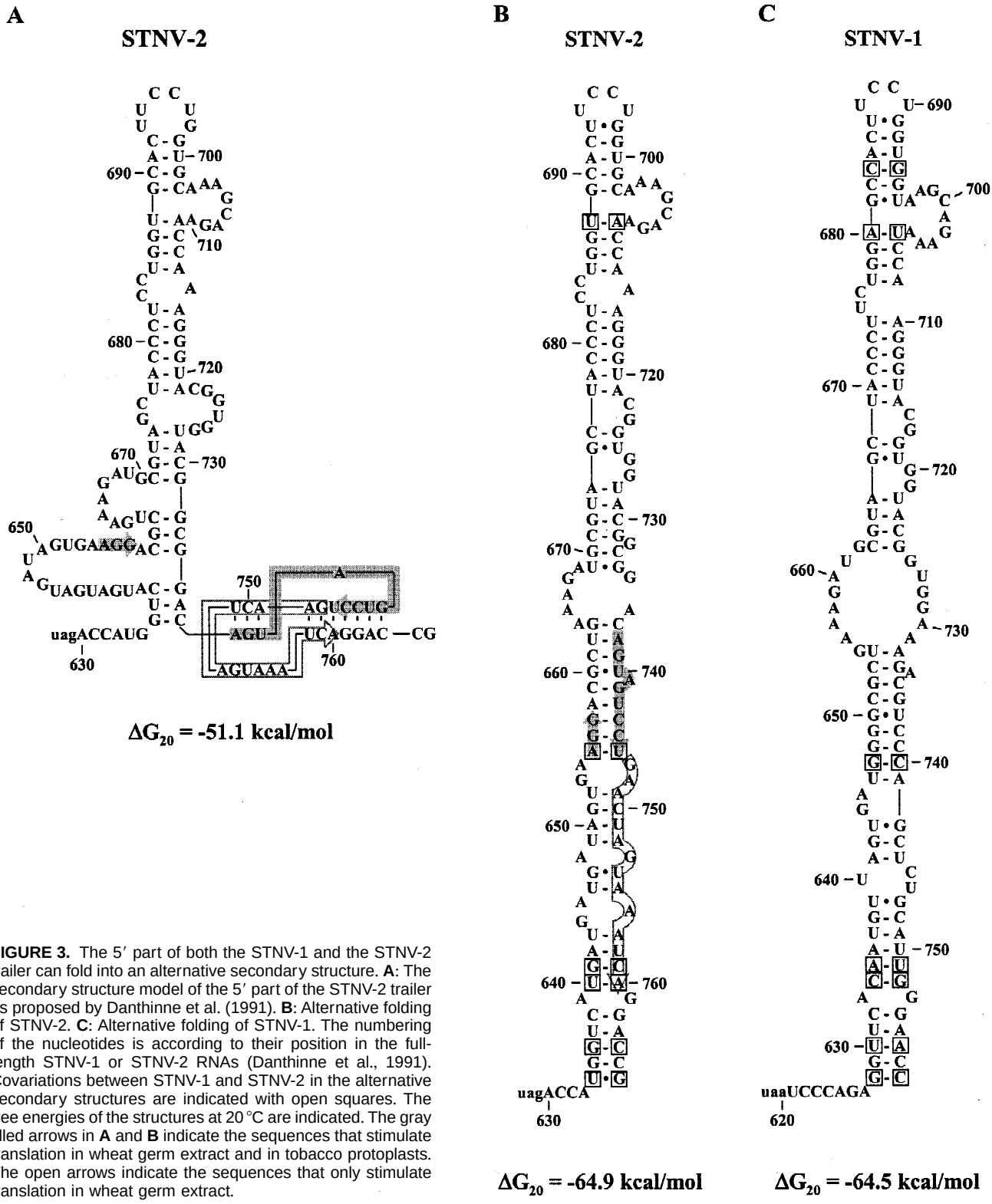


FIGURE 3. The 5′ part of both the STNV-1 and the STNV-2 trailer can fold into an alternative secondary structure. **A**: The secondary structure model of the 5′ part of the STNV-2 trailer as proposed by Danthinne et al. (1991). **B**: Alternative folding of STNV-2. **C**: Alternative folding of STNV-1. The numbering of the nucleotides is according to their position in the full-length STNV-1 or STNV-2 RNAs (Danthinne et al., 1991). Covariations between STNV-1 and STNV-2 in the alternative secondary structures are indicated with open squares. The free energies of the structures at 20 °C are indicated. The gray filled arrows in **A** and **B** indicate the sequences that stimulate translation in wheat germ extract and in tobacco protoplasts. The open arrows indicate the sequences that only stimulate translation in wheat germ extract.

ture is not sufficient for translation stimulation in both wheat germ extract and tobacco protoplasts.

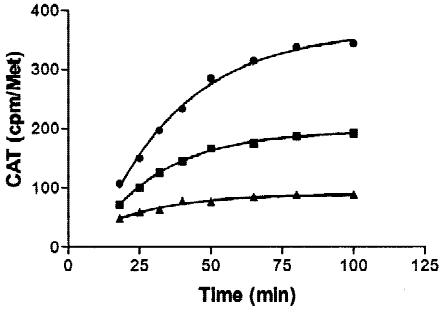
Functional 3′ TED sequences have base-pairing potential with essential 5′ TED sequences, allowing an extension of the predicted stem structure

In the secondary structure model of Danthinne et al. (1991), the STNV-2 sequences downstream of nt 737 that are required for maximal translation stimulation are part of a pseudoknot (Fig. 3A). The formation of this pseudoknot is, however, unlikely to be important for translation, as (1) not all sequences within this pseudoknot are required for translation stimulation, and (2) the pseudoknot cannot be formed in the TED derivatives that have a maximal translation stimulatory capacity. These observations let us revisit the secondary structure model and to evaluate whether TED might fold into an alternative secondary structure. Computer analysis revealed that nt 738–766, which are located downstream of the predicted stem-loop structure, have base-pairing potential with nt 634–662, which comprise the first bulge of the stem-loop (Fig. 3B). A similar base-pairing capacity exists for the STNV-1 RNA (Fig. 3C). This base pairing would result in the formation of an extended stem structure with a lower free energy than the pseudoknot-containing structure (Fig. 3A,B). Both the pseudoknot-containing structures (Danthinne et al., 1991) and the extended stem-loop structures (Fig. 3B,C) are supported by covariations. Interestingly, three bases in STNV-1 (nt 630, 740, and 753) and four in STNV-2 (nt 636, 746, 759, and 760) covary with other bases in the pseudoknot-containing structure than in the extended stem-loop structure, showing the existence of coupled covariations. This phylogenetic analysis therefore suggests that not only the pseudoknot-containing structure, but also the extended stem-loop is likely to exist. For several RNAs, mutually exclusive secondary structures requiring conformational switches have been described (Gultyaev et al., 2000). Such conformational switches may alter the functionality of the involved sequences, for example, in the case of alfalfa mosaic virus from encapsidation to replication (Olsthoorn et al., 1999). A similar scenario may be true for TED: When adopting the pseudoknot-containing structure, it may play a role in another step of the viral life cycle than when adopting the extended stem-loop structure. A first indication for a role of one of these structures in translation can be deduced from sequence requirements for TED function. In the proposed alternative structure of Figure 3B, the essential 5′ nt 655–657 base pair with 3′ located nt 744–746 that enhance translation. Moreover, extension of TED with nt 761–768, which is likely to stabilize the pseudoknot, reduces translation (Fig. 2A). This suggests that the extended stem-loop structure, rather than the pseudoknot-containing structure, may play a role in translation.

The basis of the extended stem is not essential for translation stimulation by TED 3′ sequences in wheat germ extract

Nucleotides 738–760 have a quantitative effect on translation in wheat germ extract when the 5′ end of TED is nt 632 (Fig. 2A), and thus when the extended stem as proposed in Figure 3B can be formed. If the stimulatory effect of the 3′ nucleotides were solely the result of an extension of this stem, nt 747–760 would not have a stimulatory effect when sequences upstream of nt 655 are lacking. To test whether this is the case, we translated RNAs with TED derivatives having nt 655 as 5′ end, and nt 746, 753, or 760 as 3′ end in the wheat germ extract.

Figure 4 shows that, when nt 655 is the 5′ end of TED, nt 747–760 stimulate translation about 3.5-fold. Thus, nt 632–654 are not required for the stimulatory effect of nt 747–760. The sequences upstream of $TED_{(655–753)}$ are, together with nt 747–760, not able to form a stem structure similar to that shown in Figure 3B (data not shown). This implies that with respect to the



	TE	$t_{1/2}$
• $TED_{(655-760)}$	12.22 ± 0.52	20.9 ± 1.2
■ $TED_{(655-753)}$	8.06 ± 0.21	16.9 ± 0.5
▲ $TED_{(655-746)}$	3.67 ± 0.58	17.0 ± 3.1

FIGURE 4. Nucleotides 747–760 do not require complementary 5′ sequences to stimulate translation. Uncapped *cat* RNAs with different TED derivatives were translated in the wheat germ extract. The translational efficiency (TE) is given in cpm/Met·min, and the functional half-life ($t_{1/2}$) in minutes.

stem-loop as predicted in Figure 3B, formation of the lower part is not essential for nt 747–760 to stimulate translation. Importantly, nt 747–760 stimulate translation only in wheat germ extract and not in tobacco protoplasts.

For nt 738–746, the situation is different in two respects. First, these nucleotides stimulate translation in both systems, suggesting differences in *trans* requirements between nt 738–746 and nt 747–760. Second, nt 738–746 have a base-pairing ability with the essential nt 655–657, which are also functional in both systems. This notion opens the possibility that the "middle" part of the stem-loop structure may play a role in translation both in vitro and in vivo. This part of the stem might itself be required for translation, but it might also be required for stabilization of the upper part of the stem structure. Importantly, the possible differences in structural requirements of nt 655–657 and 737–746 on the one hand, and nt 747–760 on the other hand, correlates with the presumed differences in requirement of *trans*-acting factors. Taken together, these results may suggest that TED contains two functional domains that have different *trans* requirements. One of these domains may require formation of the alternative secondary structure and is functional in vitro and in vivo. The other functional domain does not involve the alternative secondary structure and is only functional in vitro.

MATERIALS AND METHODS

Plasmid constructions

pFM136 is described by Meulewaeter et al. (1992). pFM169 and pFM191A are described by Meulewaeter et al. (1998a). pFM191B is described by Meulewaeter et al. (1998b). pRvLR655TED, pRvLT655TED, pRvLT658TED, and pRvLT 688TED are described in van Lipzig et al. (2001). pRvLR 632TED, pRvLR656TED, pRvLR657TED, pRvLR658TED, and pRvLR688TED were made using the same strategy as for pRvLR655TED (van Lipzig et al., 2001) but with the upstream primers JonecP1 (5′-CTATGTAGCTAGCCATGGTCATGATG ATGATAGT-3′), RL30 (5′-CTATGTAGCTAGCGGACGCTGA AAGATGCGTAG-3′), RL31 (5′-CTATGTAGCTAGCGACGCT GAAAGATGCGTAGC-3′), RL28 (5′-CTATGTAGCTAGCACG CTGAAAGATGCGTAGCT-3′), and JonecP2 (5′-CTATGTA GCTAGCTGCACTTCCTGGTGCAAAGC-3′), respectively. pRvLT632TED was made using the same strategy as pRvLT655TED with the upstream primer JonecP1. pRvLR 657C is identical to pRvLR655TED, but contains a G-to-C substitution at position 657 of STNV-2.

In vitro transcription

In vitro transcription of capped and uncapped RNAs was performed essentially as described by Meulewaeter et al. (1998b). The templates for the synthesis of the RNAs containing the vector-derived leader and $TED_{(632–753)}$ (of the 5′ deletion series), $TED_{(655–753)}$, $TED_{(656–753)}$, $TED_{(657–753)}$, $TED_{(658–753)}$, $TED_{(688–753)}$, or $TED_{(657C)}$ were synthesized via PCR as described by van Lipzig et al. (2001) on the templates pRvLR632TED, pRvLR655TED, pRvLR656TED, pRvLR657TED, pRvLR658TED, pRvLR688TED, and pRvLR657C, respectively. The templates for the synthesis of the RNAs with the TMV leader, and $TED_{(632–753)}$ (of the 5′ deletion series), $TED_{(655–753)}$, $TED_{(658–753)}$, or $TED_{(688–753)}$ were synthesized using the same strategy on the templates pRvLT632TED, pRvLT655TED, pRvLT658TED, and pRvLT 688TED, respectively. The templates for synthesis of the RNAs with $TED_{(632–737)}$, $TED_{(632–740)}$, $TED_{(632–743)}$, $TED_{(632–746)}$, $TED_{(632–753)}$ (of the 3′ deletion series), $TED_{(632–760)}$, $TED_{(632–764)}$, or $TED_{(632–768)}$ were synthesized via PCR with pFM191B as template for the vector-derived leader, and pFM191A for the TMV leader, with Forw18nt as upstream primer and, as downstream primer, RL15 (5′-GTCCGCCG TACCACCG-3′), RL19 (5′-ACTGTCCGCCGTACCAC-3′), RL20 (5′-ACTACTGTCCGCCGTACC-3′), RL18 (5′-AGGAC TACTGTCCGCCG-3′), RL17, RL21 (5′-TGATTTACTAGTTC AGGACTA-3′), RL23 (5′-GTCCTGATTTACTAGTTCAG-3′), and RL24 (5′-CCCGGTCCTGATTTACTAG-3′), respectively. Templates for the synthesis of the RNAs with the vector-derived leader, the *cat* coding region and $TED_{(655–746)}$ or $TED_{(655–760)}$ were PCR products on pRvLR655TED with, as upstream primer, Forw18nt, and, as downstream primer, RL18 and RL21, respectively. Templates for the RNAs with the control trailer were *Sal*I-digested pFM136 (vector-derived leader) and *Sal*I-digested pFM169 (TMV leader). Template for the RNA with vector-derived leader and without trailer was *Bsp*HI-digested pFM191B. Prior to in vitro transcription, possible inhibiting proteins or RNases were removed by treatment of the templates with Proteinase K.

In vitro translation

In vitro translation reactions (Meulewaeter et al., 1998b) were performed in the presence of [^{35}S]-methionine at 25 °C. Protein synthesis was quantified by excision of the protein bands from the gel and quantification by scintillation counting, or using a Storm 820 PhosphorImager (Molecular Dynamics) and ImageQuaNT 5.0 software. Protein accumulation (P) in function of time (t) was analyzed using the mathematical description as described by Danthinne et al. (1993):

$$P(t) = (aR_0/b)(1 - e^{-b(t-T)}) \quad (1)$$

in which T corresponds to the time point at which the first translation product is completed, a is the translational efficiency of the mRNA (= protein molecules synthesized per mRNA molecule per time unit at $t = T$), R_0 is the initial RNA input (at $t = 0$), and b is a constant that is inversely proportional to the functional half-life of the mRNA (= $t_{1/2}$) according to the relation $t_{1/2} = \ln 2/b$ (Danthinne et al., 1993). The functional half-life is the time in which the protein accumulation rate halves and thus measures the stability of the mRNA that is actively translated, as opposed to the chemical stability that measures the physical integrity of the transcript. As in these experiments the input of translatable mRNA is equal under all conditions, the product $aR_0 = A$ (= protein synthe-

sis rate at $t = T$) also reflects the translational efficiency of the mRNA. Equation (1) can also be written as:

$$P(t) = (A \cdot t_{1/2}/\ln 2)(1 - e^{-(\ln 2/t1/2)(t-T)}). \quad (2)$$

From Equation (2), it can be deduced that $P(\infty) = (A \cdot t_{1/2})/\ln 2$, showing that the protein peak level is proportional to both the translational efficiency and the functional half-life of the mRNA. By nonlinear regression using Equation (2) and the GraphPad Prism 3.0 software, a best fitting curve to the experimental data points was calculated and values for A, $t_{1/2}$, and T were obtained. The translational efficiency as shown in the figures corresponds to A.

Determination of translation and RNA stability in tobacco protoplasts

RNA introduction was done essentially as described by Meulewaeter et al. (1998a), typically introducing 15 pmol of synthetic *cat* transcript in 10^6 tobacco mesophyll protoplasts. Time point samples contained 2.5 or 3.5×10^6 protoplasts for protein or RNA analysis, respectively. Absolute CAT protein levels were determined by comparison of CAT activities with the activity of a dilution series of purified CAT protein in the presence of control protein extract.

Secondary structure analysis

The algorithm used to determine the alternative secondary structures is described in Gultyaev et al. (1995). The calculations were performed using the version 2.3 free energy parameters of D. Turner and coworkers (http:/www.ibc.wustl.edu/~zuker/rna/energy/index/shtml; after the work had been finished, this website was changed to: http://bioinfo.math.rpi.edu/~zukerm/rna/energy/index/shtml). The pseudoknot free energy parameters were estimated as described by Gultyaev et al. (1999).

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